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14. ABSTRACT

Gliomas are the most common and most deadly solid tumors that affect children. Treatment options are limited and cure rates are dismal. My laboratory has established that Mer and Axl receptor tyrosine kinase are aberrantly overexpressed in gliomas, and that inhibition of these RTKs leads to increased glioma cell apoptosis, decreased tumor cell survival and profoundly improved chemosensitivity. However, I have also recognized that Mer and Axl inhibition is associated with increased autophagy, and most recently we have found this is true in several glioma cell lines, including one of pediatric origin. I hypothesize that Mer and Axl RTK signaling regulates autophagy pathway activation in glioma cells, and this regulation determines the efficiency of glioma cell killing. We have found that Mer signaling downregulation in combination with inhibition of late stage autophagy cycling leads to decreased colony formation at three weeks in soft agar. Understanding the effects of autophagy manipulation in the setting of tyrosine kinase inhibition will help us design the most appropriate approach to targeted therapeutics for glioma and allow us to develop a clinical trial that adequately addresses both apoptosis and autophagy.

15. SUBJECT TERMS
Receptor tyrosine kinase, apoptosis, MerTK, Axl, autophagy, glioma, astrocytoma

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Amy K. Keating
DOD TNIA Annual Report
August 28, 2012

INTRODUCTION:

Gliomas are the most common and most deadly solid tumors that affect children. Treatment options are limited and cure rates are dismal. My laboratory has established that Mer and Axl receptor tyrosine kinase are aberrantly overexpressed in gliomas, and that inhibition of these RTKs leads to increased glioma cell apoptosis, decreased tumor cell survival and profoundly improved chemosensitivity. However, I have also recognized that Mer and Axl inhibition is associated with increased autophagy. Based on this new discovery, **I hypothesize that Mer and Axl RTK signaling regulates autophagy pathway activation in glioma cells, and this regulation determines the efficiency of glioma cell killing.** Better understanding of the role autophagy plays in pediatric glioma and its interaction with RTK inhibition and apoptotic pathway activation will enable us to develop efficacious clinical trials for pediatric glioma that include the appropriate manipulation of autophagy.

BODY:

Copied below is the statement of work (from 4/22/11) with comments (*in italics*) regarding details of progress and results.

Statement of Work (v. 4/22/11)

		Year 1				Year 2				Year 3			
	Task	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Specific Aim 1	1	1a			1b	1b	1b						
	2	2a	2b				2a	2b					
	3		3a	3a						3a			
Specific Aim 2	4	4a	4a	4b		4c	4d						
	5							5a	5a	5b	5c	5d	
Specific Aim 3	6	6	6										
	7			7a	7a	7a			7b	7b	7b	7c	7c
	8			8a	8a	8a			8b	8b	8b	8c	8c
Publication #1	9						9a	9b					
Publication #2	10											10a	10b

*All work proposed for this project will be performed at the University of Colorado Anschutz Medical Campus Research Complex

SA1: To test the hypothesis that Mer/Axl RTK inhibition results in increased levels or activity of known effector molecules of autophagy.

Task 1: Inhibit Mer or Axl RTK expression/signaling in G12, A172, U251, U118, and SF188 glioma cell lines.

1a. Transduction of constitutive and doxycycline-inducible shMer or shAxl into U251, U118 and SF188 cells. This has already been completed for G12 and A172 cells, needs to be done for U251, U118, SF188. [Months 1-3]

Update Aug 2012--- U251 and SF188 cell lines have been transduced with Mer and Axl constitutive shRNA, single cell sorted, and use of clonal lines for further tasks is underway [Figure 1]. U118 were not transduced, instead we chose to knockdown Mer and Axl RTK in the U87 cells as we have been successful with this cell line in in vivo murine

modeling, however this transfection failed. We will be repeating this in the next few months.

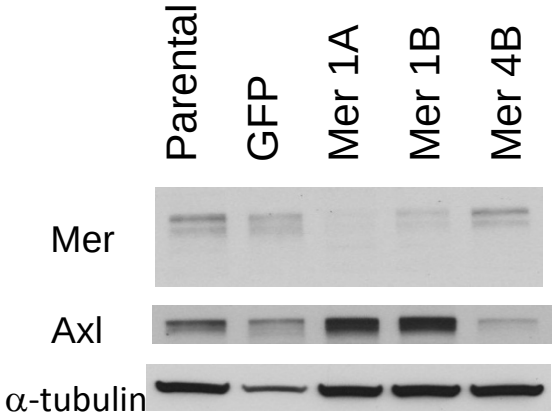

Figure 1. The U251 glioma cell line was transduced with two distinct shRNA constructs against both MerTK (shown) and Axl as well as GFP as a non-silencing control. Whole cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with an anti-Mer antibody (top), anti-Axl (middle), or anti-tubulin as a protein loading control.

1b. Apply ligand sequestration, antibody treatment or small molecule inhibitors to all cell lines as alternate modes of inhibition. [Months 10-18]

<u>Update Aug 2012---</u> We have begun inhibition of Mer RTK signaling with a small molecule inhibitor and have successfully decreased the phosphorylation of Mer [Figure 2]. We plan to test a variety of other inhibition methods in the near future.

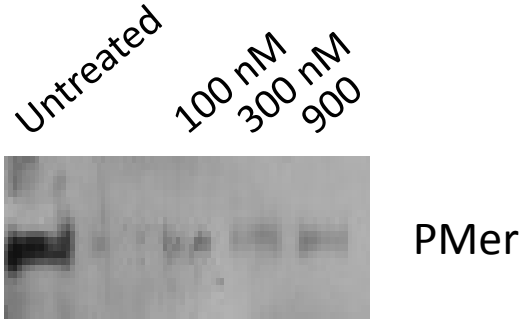

Figure 2. The parental SF188 glioma cell line was treated with a Mer/Axl specific small molecule tyrosine kinase inhibitor for 30 minutes. Whole cell lysate was immunoprecipitated with an anti-MerTK specific antibody, resolved with SDS-PAGE, transferred to nitrocellulose and then immunoblotted with an anti P-Mer antibody.

<u>Task 2:</u> Evaluate autophagic flux following RTK inhibition.

2a. Measure p62 and BHMT levels. [Months 1-3 and 16-18]

<u>Update Aug 2012---</u> We have been using conversion of LC3I to LC3II as a marker of autophagic flux. We have found that we can successfully measure autophagic flux in G12 (1), A172, SF188 and U251 cell lines with this immunoblot method, and have again reiterated the increase in autophagic flux following Mer or Axl RTK inhibition in several different glioma cell lines [Figure 3]. We are also beginning to evaluate p62 by immunoblot also.

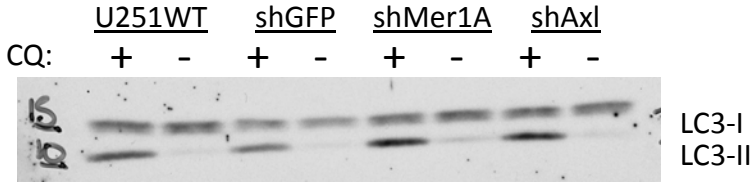

Figure 3. U251 glioma cell line wildtype (WT), control transduced (shGFP), and Mer (shMer1A) and Axl (shAxl) knockdown cells were treated with (+) chloroquine (CQ) or vehicle only (-). Whole cell lysate was resolved with SDS-PAGE, transferred to nitrocellulose and then immunoblotted with an anti LC3 antibody.

2b. Time-lapse microscopy of fluorescently tagged proteins. [Months 4-6 and 19-21]

Update Aug 2012--- We currently have GFP tagged and GFP-mCherry tagged U87 cells. We are using these to start optimization of confocal time lapse microscopy following the movement of autophagy proteins through the cell and docking of autophagosomes with the lysosome. Also we have obtained the vector to introduce this expression into other cells and have introduced them into the U251 cell line.

Task 3: Evaluate levels of autophagy effector molecules, Vps34, beclin1, ATG3, ATG5, ATG7, ATG12, Lamp2 and the Rab7 GTPase.

3a. qRT-PCR evaluation of transcript levels and immunoblot for protein levels. [Months 4-9 and 25-27]

Update Aug 2012--- We have not begun to evaluate these effector molecules but will in the upcoming months.

SA2: To test the hypothesis that Mer/Axl RTK inhibition in combination with manipulation of autophagy will lead to greater glioma tumor cell killing efficiency in vitro.

Task 4: Our working hypothesis is that Mer or Axl RTK inhibition in combination with autophagy inhibition will result in the largest tumor cell kill. Therefore we will begin with constitutive shMer and Axl knockdown G12 and A172 (already available) glioma cells and treat with (and without treatment as control) chloroquine to inhibit autophagy.

4a. Evaluate cell survival following above with MTT, develop IC50 with 95% CI. Evaluate long term non-adherent colony numbers and size with soft agar. Statistically compare constitutive shMer/Axl knockdown cells treated versus untreated by chloroquine. [Months 1-6]

Update Aug 2012--- We have plated a variety of cell lines, including U251, A172 and G12, with constitutive Mer and Axl inhibition in soft agar and treated with and without chloroquine. We have found most recently that A172 Mer KD (shMer) cells with autophagy inhibition with chloroquine treatment profoundly inhibited growth compared to those not [Figure 4], this same autophagy inhibition effect has not been noted in the parental or shGFP control transduced lines. Further analysis in this and other lines is ongoing.

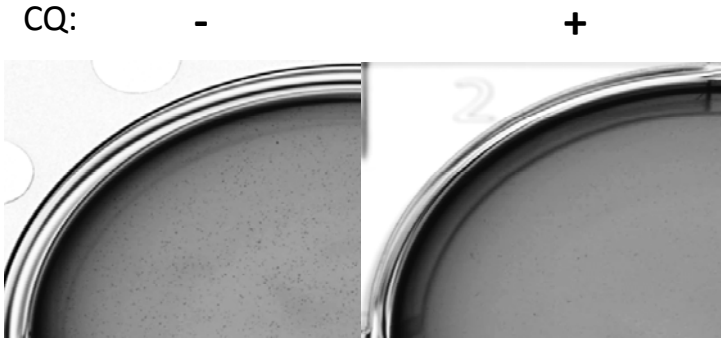

Figure 4. U251 glioma cell line transduced with Mer shRNA were plated in soft agar and treated with vehicle only (-) or chloroquine (CQ +) twice weekly for 3 weeks. Cells were then stained and counted.

4b. Apply additional phamocological methods of autophagy inhibition, including 3-MA, and Bafilomycin A1 and repeat assessments as described in 4a. [Months 7-9]

Update Aug 2012--- We have not started to investigate other pharmacologic methods of autophagy inhibition.

4c. Apply genetic methods of autophagy inhibition using siRNA directly targeting those effectors of autophagy found to be upregulated in SA1 and repeat assessments as described in subtask 4a. [Months 13-15]

4d. Test or reiterate important findings in additional cell lines once subtask 1a completed. [Months 16-18]

Task 5: Our working alternate hypothesis is that Mer or Axl RTK inhibition in combination with autophagy upregulation will result in the largest tumor cell kill. Therefore we will begin with constitutive shMer and Axl knockdown G12 glioma cells and treat with (and without treatment as control) rapamycin to increase autophagy.

5a. Evaluate cell survival following above with MTT, develop IC50 with 95% CI. Evaluate long term non-adherent colony numbers and size with soft agar. Statistically compare constitutive shMer/Axl knockdown cells treated versus untreated by rapamycin. [Months 19-24]

5b. Apply additional phamocological methods of autophagy upregulation, including trehalose and repeat assessments as described in 5a. [Months 25-27]

5c. Apply genetic methods of autophagy upregulation using induced expression of Atg1 or activated DAP kinase and repeat assessments as described in subtask 5a. [Months 28-30]

5d. Test or reiterate important findings in additional cell lines once subtask 1a completed. [Months 31-33]

SA3: To test the hypothesis that Mer/Axl RTK inhibition in combination with autophagy manipulation will lead to less in vivo glioma tumor growth.

Task 6: Write and submit animal protocol for the USAMRMC Office of Research Protection.

Update Aug 2012---Completed, submitted and approved.

Task 7: Initial in vivo investigations will be designed taking into consideration results from SA1 and SA2. Our current working hypothesis for these investigations is that Mer or Axl RTK inhibition with autophagy inhibition will prevent in vivo tumor growth most significantly; therefore we will begin with that combination.

7a. Develop SQ murine glioma models using parental (already completed) and constitutive shMer and Axl knockdown G12 and A172 (currently available) glioma cells and treat mouse with (and without treatment as control) chloroquine to inhibit autophagy. Measure tumor growth (mm3) in chloroquine treated and untreated mice by external caliper measurement and luciferin tagged imaging. Statistically compare volumes over time between groups. [Months 7-15]

Update Aug 2012--- We have successfully developed the luciferase tagged U87 and U251 cell lines and used them to develop human glioma tumors in the subcutaneous tissues of mice [Figure 5].

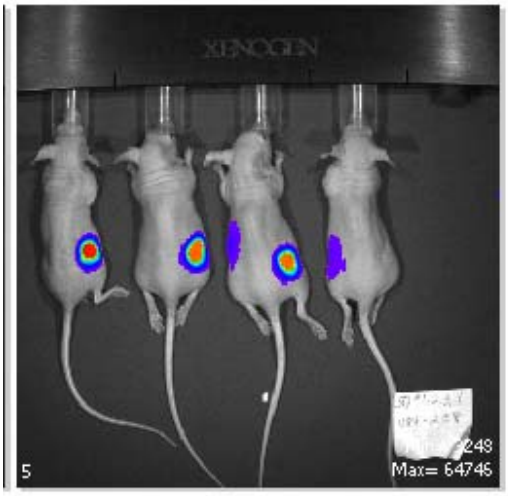

Figure 5. The U87-luciferase tagged glioma cell line was injected into the right subcutaneous flank of three nude athymic mice, while a fourth mouse was injected with carrier only (far right). Mice were imaged by IVIS following intraperitoneal injection of luciferin.

7b. Develop orthotopic frontal lobe murine glioma models using parental and constitutive shMer and Axl knockdown G12 and A172 (currently available) glioma cells and treat mouse with (and without treatment as control) chloroquine to inhibit autophagy. Measure tumor growth (mm3) in chloroquine treated and untreated mice by luciferin tagged imaging and MRI. Statistically compare volumes between groups. [Months 22-30]

Update Aug 2012--- We have successfully developed an intracranial orthotopic xenograft of human glioma using both U87 and U251 cell lines [Figure 6].

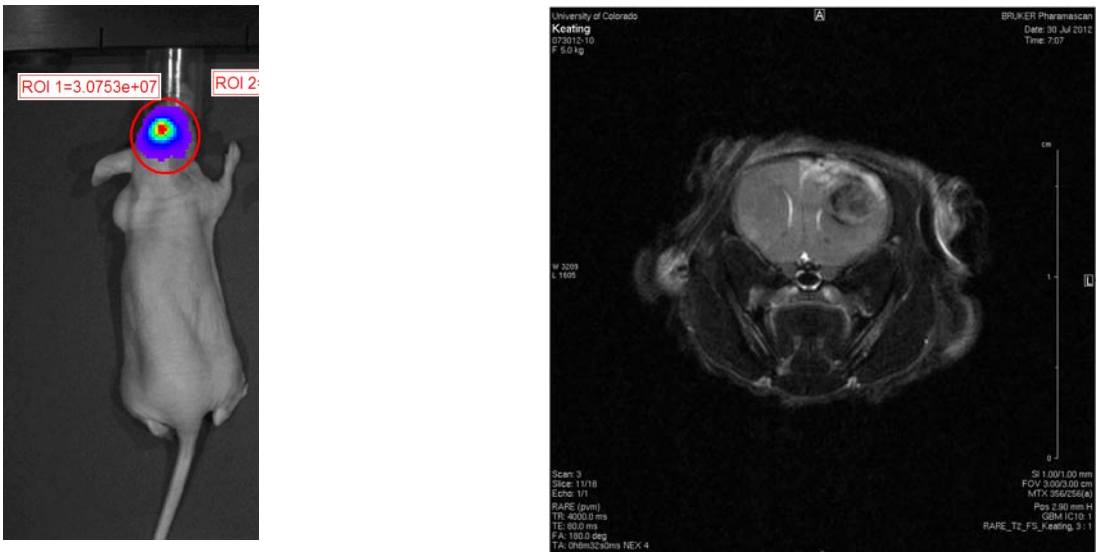

Figure 6. The U251-luciferase tagged glioma cell line was injected into the intracranial brain parenchyma in a fronto-temporal location of a nude athymic mouse. Malignant glioma growth is evidenced by IVIS (left) and MRI (right).

7c. Consider use of other available cell lines, additional inhibitors of autophagy, and alternate methods of RTK inhibition based on results from SA1 and SA2. [Months 30-36]

Task 8: Our current alternate working hypothesis for these investigations is that Mer or Axl RTK inhibition with autophagy upregulation will prevent in vivo tumor growth most significantly; therefore we will also evaluate this combination.

8a. Develop subcutaneous murine glioma models using parental (already completed) and constitutive shMer and Axl knockdown G12 and A172 (currently available) glioma cells and treat mouse with (and without treatment as control) rapamycin to upregulate autophagy. Measure tumor growth (mm3) in rapamycin treated and untreated mice by external caliper measurement and luciferin tagged imaging. Statistically compare volumes over time between groups. [Months 7-15]

Update Aug 2012--- See update for task 7a.

8b. Develop orthotopic frontal lobe murine glioma models using parental and constitutive shMer and Axl knockdown G12 and A172 (currently available) glioma cells and treat mouse with (and without treatment as control) rapamycin to upregulate autophagy. Measure tumor growth (mm3) in rapamycin treated and untreated mice by luciferin tagged imaging and MRI. Statistically compare volumes between groups. [Months 22-30]

Update Aug 2012--- See update for task 8b.

8c. Consider use of other available cell lines, additional activators of autophagy, and alternate methods of RTK inhibition based on results from SA1 and SA2. [Months 30-36]

Task 9: Peer-reviewed manuscript describing the effects of Mer/Axl RTK inhibition on autophagy activation, molecular signaling, and cellular survival.

9a. Preparation and submission [Months 16-18]

9b. Revision and publication [Months 19-21]

Task 10: Peer-reviewed manuscript describing the effects of Mer/Axl RTK inhibition in combination with autophagy manipulation and proof of principle for clinical trial development and translational applications.

10a. Preparation and submission [Months 30-33]

10b. Revision and publication [Months 33-36]

KEY RESEARCH ACCOMPLISHMENTS:

-Transduction of U251 and SF188 glioma cell lines with shRNA against Mer and Axl, which can be used in all further tasks.
-Successful inhibition of MerTK with translational small molecule inhibitors, which can be used in all further tasks.
-Optimization of autophagy flux evaluation using LC3II conversion ratios.
-Development of both a subcutaneous and intracranial xenograft model of human glioma for preclinical testing of our hypothesis.

REPORTABLE OUTCOMES:

-Previously we had found that autophagic flux was upregulated following both MerTK and Axl shRNA inhibition in the G12 cell line. We have now found that this regulation of autophagy occurs in several human glioma cell lines, including a pediatric glioma line, SF188.
-We have developed cell lines with GFP tagged LC3 as well as tanden GFP-mCherry tagged LC3-lysosome, that allow us to analyze, in real time, trafficking of autophagosomes in the live cell and their docking with the lysosome.
-Mouse model of human glioma, both subcutaneous and intracranial, allowing us to manipulate both apoptotic signals and autophagy and follow the effects in an in vivo model.

CONCLUSION:

Mer and Axl RTKs regulate autophagic flux in human glioma cell lines. We hypothesize that this results in cell survival and that through inhibition of this upregulation we can improve malignant cell death. Understanding the effects of autophagy manipulation in the setting of tyrosine kinase inhibition will help us design the most appropriate approach to targeted therapeutics for glioma.

REFERENCES:

1. A. K. Keating *et al.*, Inhibition of Mer and Axl receptor tyrosine kinases in astrocytoma cells leads to increased apoptosis and improved chemosensitivity. *Mol Cancer Ther* **9**, 1298 (May, 2010).

APPENDICES: None